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Abbreviations

A:P	animal protein-to-potassium ratio
ALT	alanine aminotransferase
ANOVA	analysis of variance
BMI	body mass index
CI	confidence interval
DAL	dietary acid load
DQ	dietary quality
FFQ	food frequency questionnaire
GFR	glomerular filtration rate
HOMA-IR	homeostasis model assessment of insulin resistance
MET	metabolic equivalent task
NAFLD	non-alcoholic fatty liver disease
NEAP	net endogenous acid production
OR	odds ratio
PRAL	potential renal acid load
Q	quartile
RS	Rotterdam Study

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Objective
Our group recently showed that animal protein was independently associated with non-alcoholic fatty liver disease (NAFLD). We hypothesize that this may be explained by a high diet-dependent acid load (DAL).
Methods
This cross-sectional study is embedded in a prospective population-based cohort. We estimated DAL-proxies via food-frequency questionnaires using potential renal acid load (PRAL; using dietary protein, phosphorus, potassium, calcium, and magnesium intake), net endogenous acid production (NEAP; using protein and potassium intake), and animal-protein-to-potassium-ratio (A:P). We defined NAFLD using ultrasound after excluding secondary steatogenic causes. We used logistic regression models –adjusted for socio-demographic, lifestyle, and metabolic traits– on categorized (Q1-Q4) and continuous DAL-proxies (allowing for non-linearity) and NAFLD.
Results

We included 3882 participants of which 1337 had NAFLD. All DAL-proxies were higher, meaning more acidic, in individuals with NAFLD (PRAL: -2.9 vs -5.5mEq/day; NEAP: 37.0 vs 35.1mEq/day, and AP:13.3 vs 12.4; all P<0.001). The highest quartile of DAL-proxies was associated with NAFLD independent of socio-demographic and lifestyle confounders, but significance dissipated after correction for metabolic confounders and multiple testing. However, the P-value for non-linearity was significant in all DAL-proxies (P<0.001). Natural cubic splines performed better with than without DAL-proxies in the fully adjusted model (all P≤0.038). The highest probability of NAFLD was found for an acidic diet.
Conclusions
This study showed an independent non-linear association between an acidic diet and NAFLD. Further studies with acid-base biomarkers are needed, but our findings might provide a mechanistic explanation for the harmful association between an animal protein-rich diet and NAFLD.

In this population-based cohort (n=3882), there is a non-linear, independent association between dietary acid load & NAFLD, which may explain the previous found link between animal protein & NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease worldwide with an estimated prevalence of 25% in the adult population(1). Its occurrence closely parallels the obesity epidemic. Obesity and insulin resistance are therefore reckoned as novel risk factors for liver disease in absence of the traditional risk factors, i.e. alcohol misuse or viral hepatitis(2). NAFLD can progress to more severe liver disease with hepatic fibrosis or cirrhosis potentially leading to need for transplantation or even liver-related death(3). In addition, NAFLD is a major risk factor for incidence cardiovascular disease(4), and indeed the most common cause of death in NAFLD is related to cardiovascular events(5). Given the above, it is of great public health interest to unravel NAFLD pathophysiology in order to improve understanding and treatment thereof.

Adhering to a healthy lifestyle, in terms of implementing a well-balanced diet and effectuating adequate physical activity, is the cornerstone of treatment of NAFLD across the entire spectrum of the disease(6,7). Our contemporary Western diet, on the other hand, is related with an increased risk of NAFLD development(8). This Western diet typically consists of a high intake of animal-based food products and sugar-containing beverages, and by low intake of fruit, vegetables and whole grains(9). Our group recently showed that animal protein was the only (subtype of) macronutrient that was independently associated with higher prevalence of NAFLD in an elderly population-based cohort(10). Interestingly, others have found that red meat intake was associated with increased overall mortality, and in particular with liver-related mortality(11). However, the underlying mechanisms that contribute to this association remain elusive.

It has been previously postulated that a Western diet may cause low-grade metabolic acidosis, which may subsequently lead to metabolic disturbances such as type 2 diabetes(12) and cardiovascular diseases(13). The rationale for this hypothesis is that this diet is rich in food items that supply acid precursors (i.e. non-carbonic acids such as sulfuric acid from meat and fish) and low in food items that supply base precursors (i.e. alkali salts from organic acids such as citrate and bicarbonate from vegetables and fruits), leading to a disturbance in acid-base balance(14,15). Two preceding studies have suggested an association between diet-dependent acid load (DAL) and NAFLD, independent of body mass index (BMI)(16,17). Amongst them, Krupp et al. showed that potential renal acid load (PRAL, a proxy of DAL), was associated with alanine aminotransferase (ALT) and steatosis, as defined by a surrogate diagnostic algorithm, in a small study of healthy adolescents(17). Moreover, Chan and

colleagues demonstrated that net endogenous acid production (NEAP, another proxy of DAL) but not PRAL, was associated with MRI-diagnosed NAFLD in a Chinese population (n=793)(16). In addition, two recent Japanese studies have showed an association between low urine pH (indicator of metabolic acidosis) and incident NAFLD(18,19).

To date, however, there has been no large-scale study on the association of diet-dependent acid load and NAFLD in Western adults. We therefore aimed to evaluate if DAL (as assessed by PRAL, NEAP, and animal protein-to-potassium ratio [A:P]), was independently associated with ultrasound-defined NAFLD in an elderly Western population.

Subjects and Methods

Study Population

This study is embedded in The Rotterdam Study, a prospective cohort study, that was initiated in the mid-1980s in order to study our aging population in more detail(20). The design and rationale behind The Rotterdam Study have been described in more detail previously(10,20) and is also described in the **Supplementary Methods I**(21). In short, all participants of The Rotterdam Study reside in Ommoord, a suburb of Rotterdam, the Netherlands, and all participants were aged 45 or 55 years or above at time of first enrollment. The Rotterdam Study consists of three different cohorts (RS I, RS II, and RS III) that each visited the research centre multiple times. From 2009 onwards the hepatology department joined this research initiative by expanding the extensive physical work-up with liver imaging (this comprises cohort RS I-5, RS II-3, and RS III-2). The Rotterdam Study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus MC University Medical Centre Rotterdam and by the review board of The Netherlands Ministry of Health, Welfare, and Sports. Written informed consent was obtained from all participants.

Dietary data and diet-dependent acid load

We requested all participants to complete an externally validated, semi-quantitative, 389-item food frequency questionnaire (FFQ) that was developed for Dutch adults(22,23). Habitual dietary intake was assessed by means of detailed questions on food item consumption over the last month that addressed not only type of food, but also quantity, portion size, and preparation methods. Servings were estimated in grams or in milligrams per day using standardised household measures(24). We extracted macronutrient intake from the questionnaires using the Dutch Food Composition Table (NEVO v2011) that contains information on nutrient content per gram or serving per product. DAL was calculated using three previously defined algorithms, which we will refer to as DAL-proxies from this point forward. The DAL-proxies included: 1) potential renal acid load (PRAL)(25), 2) net endogenous acid production (NEAP)(15), and 3) animal protein-to-potassium ratio (A:P)(26).

Remer and Manz developed PRAL to proxy the renal net acid excretion using nutrient intake data(25). PRAL was validated against urine pH in 24 hour urine samples from 63 healthy volunteers (25).

$$\begin{aligned}\text{PRAL}\ (\text{mEq/day})(25) = \quad & 0.4888 \times \text{protein}\ [\text{g/day}] + \\ & 0.0366 \times \text{phosphorus}\ [\text{mg/day}] - \\ & 0.0205 \times \text{potassium}\ [\text{mg/day}] - \\ & 0.0125 \times \text{calcium}\ [\text{mg/day}] - \\ & 0.0263 \times \text{magnesium}\ [\text{mg/day}]\end{aligned}$$

Shortly after, Frassetto et al. developed a simplified algorithm (NEAP) using dietary protein as acid precursor and potassium only, as base precursor from organic anions(15). This

algorithm was validated in 141 healthy men and women consuming 20 different diets, and NEAP accounted for over 70% of the variation in renal net acid excretion.

$$\text{NEAP}\ (\text{mEq/day})(15) = \left(54.5 \times \text{protein}\ [\text{g/day}]\ /\ \text{potassium}\ [\text{mEq/day}]\right) - 10.2$$

Finally, Zwart and colleagues suggested the use of the ratio between animal protein intake and potassium intake instead of total dietary protein, as animal protein is considered to be the main contributor to diet-dependent acid load(26,27).

$$\text{A:P}(26) = \text{animal protein}\ [\text{g/day}]\ /\ \text{potassium}\ [\text{g/day}]$$

We assessed adherence to the Dutch Dietary Guidelines 2015, using a predefined index that describes a general advice to follow a balanced and healthy dietary pattern(28), in order to assess dietary quality (DQ). Briefly, this guideline comprises specified recommendations on I) vegetables(≥200g/day), II) fruit (≥200g/day), III) whole-grain products (≥90g/day), IV) legumes (≥135g/week), V) unsalted nuts (≥15g/day), VI) fish (≥100g/week), VII) dairy (≥350g/day), VIII) tea (≥150mL/day), IX) whole grains ≥50% of total grains, X) unsaturated fats and oils ≥50% of total fats, XII) red and processed meat <300g/week, XIII) sugar-containing beverages (≤150mL/day), XIV) alcohol (≤10 g/day), and XV) salt (≤6 g/day). Incomplete FFQs were excluded as well as FFQs with unreliable energy intake (i.e. <500 calories/day or ≥7500 calories/day).

Assessment of steatosis

Abdominal ultrasound was performed by a certified and experienced technician on a Hitachi HI VISION 900 (PvW). Ultrasound images were stored digitally so re-evaluation by an experienced hepatologist (RdK) was possible. All FFQs were filled in prior to liver imaging, so participants were unaware of the ultrasound results when completing the FFQ. Diagnosis of steatosis was determined dichotomously(29), measuring the hyper-echogenicity of liver parenchyma. In order to define NAFLD, we first excluded participants that used steatogenic drugs (i.e. systemic corticosteroids, methotrexate, tamoxifen or amiodarone). This was identified through linkage with pharmacy data. Second, participants with viral hepatitis were excluded, based on hepatitis B surface antigen and anti-hepatitis C virus serology which was assessed using an automatic immunoassay (Roche Diagnostic GmbH). And third, we excluded participants with excessive alcohol consumption (>30g/day for men and >20 g/day for women, based on the FFQ).

Other Covariates

We obtained data on demographics, physical activity, and education level by means of an extensive home interview by qualified interviewers. Briefly, physical activity was assessed using the LASA Physical Activity Questionnaire and expressed in metabolic equivalent of task (MET)hours/week(30). Blood pressure was measured at a single visit using two successive measurements in a sitting position, and blood samples were collected after overnight fasting. Automatic enzyme procedures were used to measure blood lipids, platelet count, glucose, ALT, aspartate aminotransferase, and gamma-glutamyltransferase (Roche Diagnostic GmbH, Mannheim, DE). An automatic immunoassay was used to determine insulin (Roche Diagnostic GmbH). Estimated glomerular filtration rate (GFR in [ml/min/1.73 m2]) was based on calibrated creatinine levels using the CKD-EPI Creatinine Equation (2009). Creatinine levels were calibrated by aligning mean values of serum creatinine from our cohort with those of the Third National Health and Nutrition Examination Survey (NHANES III) in different age and sex specific categories(31). Anthropometrics were measured by well-trained research assistants. The presence of hypertension was diagnosed if

either systolic (≥140mmHg) or diastolic (≥90mmHg) blood pressure was increased or the participant was on anti-hypertensive medication. Diabetes was defined as fasting glucose above 7.0 mmol/L (≥126mg/dL) or drug treatment for elevated blood glucose. Metabolic syndrome was diagnosed if three out of five of the following traits were present: 1) high waist circumference (≥102 cm for men and ≥88 cm for women), 2) high blood pressure (≥130/85 mmHg), 3) HDL below 1.0 mmol/L (≤40 mg/dL) in men and below 1.3 mmol/L (≤50 mg/dL) in women or the use of lipid-lowering drugs, 4) triglycerides above 1.7 mmol/L (≥150 mg/dL) in both sexes or the use of lipid-lowering drugs, and finally 5) fasting glucose above 5.6mmol/L (≥100 mg/dL)(32). Insulin resistance was calculated using the homeostasis assessment model of insulin resistance (HOMA-IR): (fasting glucose [mmol/L] x fasting insulin [mU/lL]) / 22.5(33).

Statistical Analyses

We excluded all participants with missing or unreliable FFQs, and participants with more than 30% missing study variables. Variables were imputed using multiple imputation under the fully conditioned specification to reduced bias due to missing data(34). A more detailed description on the imputation process can be found in the **Supplementary Methods II**(21).

Population characteristics of both the original and imputed data were described using the mean (standard deviation, SD), median (25^{th} and 75^{th} percentile, P25-P75), or percentage. We carried out analyses of variance (ANOVA) to compare means for different strata (NAFLD vs no NAFLD and quartiles of DAL) and Kruskal-Wallis tests to compare medians for different strata. Chi-square tests were used to compare categorical variables across strata. We calculated Spearman rank correlation coefficients in order to give more insight in the correlations of DAL with dietary macronutrients, dietary micronutrients, and DQ. We used logistic regression models to assess the association between DAL-proxy categories and NAFLD for comparability with other dietary-acid load studies that used categorical analyses (17) and to facilitate clinical interpretation. Then we assessed linearity, fitting models with splines to allow for non-linearity using natural cubic splines. Thereafter, we tested the need for the non-linear terms and optimal degrees of freedom by comparing the spline model against linear models with the Akaike Information Criterion. And lastly, we tested the relevance of DAL as predictor for NAFLD by comparing the spline model to a model without DAL using a likelihood ratio test.

We adjusted all analyses for potential confounders using 4 models. In model 1, we adjusted for potential socio-demographic confounders, i.e. age, sex, education level (low/moderate/high), study cohort (RSI/RSII/RSIII), and for energy intake (kilocalories). In model 2, we adjusted for lifestyle confounding factors such as alcohol use (in units, one units is 10grams), physical activity (MET equivalent hours/week), and smoking (current/past or never). In model 3 (the main model) we adjusted for metabolic variables, i.e. HDL-cholesterol (in mmol/L), triglycerides (in mmol/L), presence of metabolic syndrome, estimated GFR (in ml/min/1.73m^2), presence of diabetes mellitus and BMI (in kg/m^2). And finally, in model 4 we tested the potential confounding effect of DQ on the association between DAL and NAFLD. The selection of covariates was based on previous literature(35). Results were expressed as predicted probability or odds ratio (OR) per quartile, both with the accompanying 95% confidence interval (CI). In addition, we tested all models for multicollinearity (VIF>5.0). Furthermore, in order to test the robustness of our results we performed several sensitivity analyses. First, to account for potential measurement error in dietary intake and to remove extraneous variation arising from total energy intake, all DAL-proxies were adjusted for energy intake using the residual method: that part of the DAL-proxies that was not explained by total energy intake(36). Second, in order to test the robustness of our results, we performed several stratified analyses. We stratified by sex, as gender-differences in DAL have been previously suggested(12,17). We stratified by cohort,

as participants from various cohorts differ in terms of age (mean age RSIII: 62, RS II: 72, and RS I: 79 years old) and because there was a median time-gap of 5.5 years between completing the FFQ and performance of ultrasound in cohort three (RS-III, see **Supplementary Methods I**)(21). But as dietary data are known to be stable over time(37), RS-III was included in the main analysis. We also stratified by GFR, using 60 ml/min/1.73m^2 as cut-off to distinguish between a normal and impaired renal function (as the kidneys play a crucial role in maintaining acid-base balance in the body(38)). We stratified by age (using 65 as cut-off), as DAL-associations have been previously observed particularly in young individuals(39). And finally, we stratified by BMI at a cut-point of 25kg/m^2, as participants with a normal BMI (lean) could have a different pathophysiological pathway compared to overweight participants(12,40). Third, we replaced presence of diabetes with the continuous proxy for insulin resistance, HOMA-IR, in model 3, in order to test the hypothesis that the mechanistic explanation behind DAL associations are mediated by insulin resistance(41,42). And lastly, we alternately excluded the Q1 or the Q4 from the continuous spline analyses in order to assess whether it was the alkaline component or the acidic component (respectively), or both, that drives the association between DAL-proxies and NAFLD.

To correct for the inflated type I error that arises due to multiple testing we applied the method proposed by Sidák(43), adapted as described in Galwey et al.(44), using the effective number of tests (n=1.8) instead of the actual number of tests (n=3). This adaptation is necessary to take into account that dietary exposures inter-correlate instead of being independent from each other. The resulting corrected significance level for all DAL-proxy analyses was P<0.028. All analyses were performed using R version 3.5.1.

Results

Participant Characteristics

In total, 5967 participants were eligible for this study. We excluded unreliable FFQs (n=98; 1.6%) and missing FFQs (n=1075; 18%). These participants were significantly younger (68.5 vs 69.6 years; P<0.01), less often of European descent (95% vs 98%; P<0.01), and had a higher BMI (27.5 vs 26.9 kg/m^2; P<0.01), but there was no difference in steatosis prevalence (37.2% vs 35.5%; P=0.27) and sex (55.8% vs 57.5%; P=0.27). Subsequently, we excluded 40 participants (0.8%) that had >30% of missing data on study variables. Lastly, we excluded 872 participants (18.3%) with potential secondary causes for steatosis (n=123 steatogenic drug use, n=691 alcohol misuse, n=31 viral hepatitis, n=27 combination of the aforementioned factors; **Supplementary Methods I**)(21). Hence, the total study population consisted of 3882 participants of which 1337 individuals had NAFLD (34.4%). The median DAL-proxies in this population were as follows: PRAL -4.7 mEq/day (-15.4; 4.4), NEAP 35.7 mEq/day (29.6-42.3), and A:P 12.7 (10.2-15.4). Population characteristics on both original and imputed data are shown in **Supplementary Table 1**(21). In short, mean age was 69.7 years (8.8), median BMI was 26.9 (24.5-29.7), 58.3% was female, and the majority was of European descent (97.6%). In **Supplementary Table 2** population characteristics are shown according to NAFLD stratum(21). Median DAL-proxies were significantly higher in participants with NAFLD than in participants without NAFLD, i.e. for PRAL -2.9 vs. -5.5 mEq/day (P=4.97e^{-6}), for NEAP 37.0 vs. 35.1 mEq/day (P=2.74e^{-6}), and for A:P 13.3 vs. 12.4 (P=1.70e^{-10}).

Characteristics of diet-dependent acid load

Population characteristics per PRAL-quartile are given in **Table 1**. The median PRAL of Q4 was 11.1 (7.4; 17.6) mEq/day. In addition, characteristics per NEAP and A:P quartile are depicted in **Supplementary Table 3 A–B**(21). The median NEAP in the Q4 of NEAP was 47.5 (44.6-53.0) mEq/day, and the median A:P in the Q4 of A:P was 17.8 (16.4-20.1). Similar characteristics for Q4 in all DAL-proxies were found, being lower proportion of

females, lower physical activity, more current or former smokers, higher BMI and higher gamma glutamyltransferase. In addition, there were more comorbidities, in particular higher prevalence of NAFLD and diabetes (**Table 1** and **Supplementary Table 3 A - B**)(21).

PRAL, NEAP and A:P correlations with other dietary parameters are depicted in **Table 2**. All DAL-proxies correlated inversely with DQ, meaning that the higher the DAL-proxy the lower dietary quality (r_s=-0.29 for PRAL, r_s=-0.29 for NEAP, and r_s=-0.32 for A:P). In particular fruit intake had a strong inverse correlation with DAL (r_s=-0.51 for PRAL, r_s=-0.50 for NEAP, and r_s=-0.37 for A:P). Also, mono and disaccharides and fiber intake were inversely correlated (**Table 2**). As expected, all DAL-proxies correlated positively with animal protein (r_s=0.26 for PRAL, r_s=0.32 for NEAP, and r_s=0.51 for A:P).

Categorized diet-dependent acid load and NAFLD

The highest quartile (Q4) of PRAL was associated with higher prevalence of NAFLD (using Q1 as reference), in all models (**Table 3**). However, after correction for multiple testing, the association between PRAL and NAFLD was no longer significant in model 3 (OR_{Q4vsQ1} 1.26, 95%CI 1.01-1.58; *P* = 0.041). A similar association was seen for NEAP, in which the Q4 of NEAP was only significantly associated with NAFLD in model 1 and 2 (model 3: OR_{Q4vsQ1} 1.24, 95%CI 0.99-1.56; *P*=0.058). Lastly, the Q4 of A:P had a more pronounced association with NAFLD than PRAL and NEAP in the first two models, but this association was confounded by metabolic factors –in particular by the metabolic syndrome, BMI, and diabetes mellitus– in model 3 (OR_{Q4vsQ1} 1.22, 95%CI 0.97-1.52; *P*=0.089).

Continuous diet-dependent acid load and NAFLD

Logistic regression with natural cubic splines for DAL showed a clear non-linear effect of DAL (*P* for non-linearity for PRAL: $6.5 \cdot 10^{-4}$, NEAP: $3.7 \cdot 10^{-4}$, and A:P: $2.7 \cdot 10^{-5}$) . The predicted probability of NAFLD in model 3 (metabolic) was lowest within the alkaline PRAL range -45 to -4 mEq/day, with a minimum predicted probability of NAFLD that was 29%. Whereas the predicted probability of NAFLD increased to 36% for acidic PRAL-values 9 to 11 mEq/day (equals Q4; **Figure 1A**). A similar shape was seen for NEAP and A:P (**Figure 1B – C**). For NEAP Q1 and Q2 NAFLD probability was low (<33%), but increased for towards 37% (**Figure 1B**). For Q1 and Q2 of A:P probability of NAFLD was low (again <33%), and the highest predicted probability of NAFLD (36%) was seen at an A:P of 17 in (again equals Q4; **Figure 1C**). The shape of the splines for models 1 and 2 were very similar to that of model 3, but generally with higher predicted probabilities of NAFLD and more pronounced differences between high and low DAL (**Supplementary Figure 1 A – B**)(21). All models performed better with DAL in the model than without DAL, this even remained significant for NEAP and A:P after multiple comparison correction (log-likelihood ratio test: *P* = 0.038 for PRAL; *P* = 0.015 for NEAP; *P* = 0.012 for A:P).

Diet-dependent acid load and potential confounding by dietary quality

We assessed whether DAL was confounded by DQ in a separate model 4. Categorical analyses of DAL-proxies with NAFLD adjusted for DQ are depicted in **Table 3; model 4**. Although the results were not statistically significant, the associations hardly attenuated after adjustment of DQ. Hence, DQ could not fully explain the association between DAL and NAFLD. The same effect of DQ was seen on the splines (**Supplementary Figure 1C**: PRAL *P*=0.043, NEAP *P*=0.017; and A:P *P*=0.014, comparing the models with and without DAL-proxies)(21).

Sensitivity analyses

To test the robustness of our findings we carried out multiple sensitivity analyses. First, we used the residual method to account for extraneous variation in DAL arising from total energy intake. The associations with PRAL attenuated slightly, but the results for NEAP and A:P were similar to the main analysis (**Supplementary Table 4**)(21). Second, we stratified

by several predefined covariates (**Supplementary Tables 5-9**)(21). All stratified results largely resembled the original analyses (**Figure 2**). Interestingly, PRAL was nominally significant associated with higher NAFLD prevalence in participants with an impaired kidney function after full adjustment in the metabolic model (OR_{Q4vsQ1} 1.81 95%CI 1.01-3.24, *P* = 0.047, **Supplementary Table 7**)(21). Third, we have additionally adjusted model 3 for insulin resistance (instead of diabetes presence, using HOMA-IR) and found that the association dissipated, indicating the mediating role of insulin resistance in this association (OR_{Q4vsQ1} for PRAL: 1.19, 95%CI 0.95-1.50; for NEAP 1.15 95%CI 0.88-1.38; for A:P 1.12 95%CI 0.89-1.41). And fourth, in order to assess whether it is the acidic component or the alkaline component that drives the association between DAL-proxies and NAFLD, we alternately excluded the alkaline component (Q1) and the acidic component (Q4) from the analyses. All models with DAL-proxies performed better than models without DAL-proxies only when the alkaline component was excluded, but not when the acidic component was excluded (log-likelihood ratio test excluding Q1: *P*=0.032 for PRAL; *P*=0.015 for NEAP; *P*=0.010 for A:P; log-likelihood ratio test excluding Q4: *P*=0.310 for PRAL; *P*=0.115 for NEAP; *P*=0.253 for A:P) This may indicate that it is the acidic component drives the association between DAL-proxies and NAFLD.

Discussion

In this largest population-based cohort study to date, we found that diet-dependent acid load, as assessed by net-endogenous acid production and animal protein-to-potassium ratio, was independently associated with NAFLD. This association was not linear; but the highest probability of NAFLD (36-37%) was found for an acidic diet and the minimum predicted probability of NAFLD (29%) for an alkaline diet. Indeed, the association was driven mainly by the acidic component of the diet. Models with NEAP and A:P performed significantly better in predicting NAFLD than without these indices of DAL, importantly, even after correction for numerous confounders such as BMI and overall dietary quality. Moreover, the association between DAL-proxies and NAFLD was tested for in different subgroups of the population which showed overall consistent results.

Despite all ongoing pharmacological studies, lifestyle intervention remains the only available treatment for NAFLD today(45). Recently, several studies found detrimental associations between high animal protein intake (10,46) and NAFLD. Amongst them, a recent study from Zelber-Sagi and colleagues found that high red and processed meat consumption was associated with NAFLD and insulin resistance(47). This is in line with another recent study from Etemadi et al. that showed that a diet rich in red meat was associated with higher incidence of liver-related mortality(11). Food items from the Western dietary pattern, such as red meat, generally contribute to a higher diet-dependent acid load(38). Indeed, animal protein had the highest positive correlation with all DAL-proxies in our study as well. Interestingly, the highest inverse correlation with DAL-proxies was found for mono-and disaccharides. This is in line with our previous study in which we found an inverse association of mono-and disaccharides with NAFLD, though this was not independent from metabolic confounders. We also know that fruit was by far the most contributing group to this macronutrient. And fruits have indeed a alkalinizing potential(38).

In this study we show that in particular an acidic diet was associated with higher NAFLD prevalence, and we therefore hypothesize that diet-dependent acid load may (partially) explain the link between an animal protein-rich diet and NAFLD. Interestingly, our findings are in line with two previous studies on DAL and NAFLD(16,17). A study in German adolescents found that PRAL was associated with ALT, fatty liver index and hepatic steatosis index in girls but not in boys(17). However, the study population was small (n=145), the study only included adolescents and young adults (mean age 20 years old), and steatosis

algorithms were assessed only continuously (but prevalence was most probably low as median FLI was ± 10). Another elegant study analysed PRAL and NEAP in association with NAFLD (assessed by MRI-PDFF), in 793 Chinese adults(16). The authors found an association between NEAP and NAFLD in multivariable analysis. However, linearity of PRAL and NEAP was not assessed in these two studies(16,17), which makes direct comparison (along with major sociodemographic differences) to our Western adult population difficult. Of note, mean diet-dependent acid load in the latter study was rather high (PRAL 24 mEq/day and NEAP 77 mEq/day)(16) in comparison to our study and that of others which was relatively more alkaline (i.e. the Rotterdam Study: PRAL -4.7mEq/day and NEAP 36 mEq/day; Nurses' Health Study: PRAL -3.1 mEq/day and NEAP: 44 mEq/day; and Health Professionals' Follow-up Study: PRAL 5.7 mEq/day and NEAP 48 mEq/day)(12).

Diet-dependent acid load has also been implicated in other health outcomes that are related to NAFLD, such as type 2 diabetes and hypertension(38). An interesting Swedish study examined whether PRAL was associated with overall mortality in over 80.000 individuals followed for 13 years(48). Similar to our study, the authors found a non-linear association between PRAL and all-cause mortality but rather a U-shaped spline. Thus, both dietary acid and alkali excess were associated with increased mortality risk. We did not observe a clear U-shape in our splines, but the predicted probability of NAFLD was indeed also higher in the distinct alkaline diets. To date, there is no study that showed that a diet high in alkaline load is detrimental for health, however, one could speculate that a one-sided, unbalanced diet might be unhealthy anyway – possibly also via other mechanisms than diet-dependent acid load alone. Rightfully so, the authors of this Swedish study argued whether the low impact (HR 1.06) of PRAL on mortality risk is important in terms of public health. Indeed, there are studies that found no association between diet-dependent acid load and health(49,50). Differences in sociodemographic factors such as age, gender, ethnicity, and dietary habits could have contributed to these contrasting results. We have therefore performed several predefined subgroup analyses. Most subgroup analyses were not statistically significant, but should be interpreted in light of lower statistical power, and generally confirmed the main results. Yet, the highest quartile of PRAL was nominally significant in participants with an impaired renal function (estimated GFR < 60 ml/min/1.73m^2) and, albeit not significant, the same trend was seen for both NEAP and A:P. The results were not significant after multiple testing correction, but again, they should be interpreted in light of lower power (n=597). This finding is not surprising as the kidney plays a crucial role in maintaining acid-base balance(51). Likewise, a long-term high diet-dependent acid load could also contribute to the development of chronic kidney disease by increasing endothelin-1, angiotensin-II, and aldosterone production to meet the demand for hydrogen excretion(52).

Mechanistically, it is thought that consumed proteins, in particular sulphur-containing amino-acids (e.g. methionine and cysteine) found in animal proteins, form sulphate after oxidation(38). This is a non-volatile acid that can be neutralized by bicarbonate but forms a hydrogen-bond as end product. In contrast, plant protein often contains glutamate which can be metabolized without this formation. Fruit on the other hand, is potassium-rich and often accompanied by citrate and malate, which consume hydrogen-bonds to become neutral and hence are alkalinizing(53). This low-grade or subclinical metabolic acidosis has been associated with various metabolic alterations(54). Amongst others, a change in glucose homeostasis has been found, being a lower insulin response to high glucose in the presence of a low pH(41). Also, long-term subclinical metabolic acidosis may increase adrenal cortisol production (modulated by the hypothalamic-pituitary-adrenal axis) and subsequently may lead to visceral obesity and insulin resistance(42,55). In our study, adjustment for insulin resistance, as assessed by HOMA-IR, weakened the association between DAL and NAFLD,

confirming our hypothesis that the association between DAL and NAFLD is mediated via insulin resistance(41,42). Based on experimental studies, it has been proposed that low-grade metabolic acidosis influenced the growth hormone/ insulin growth factor 1 system, leading to hepatocellular growth hormone resistance and subsequent hepatic lipid accumulation(56,57). Opponents of the diet-dependent acid load hypothesis argue that DAL-proxies are merely a different way of scoring adherence to a healthy diet. Although we cannot exclude this possibility, separate correction for adherence to dietary quality did not fully explain the association between DAL and NAFLD. Indeed, food items can effectuate health changes without affecting acid-base balance. For example, fruits and vegetables also contain dietary fiber that is beneficial for glycemic control(58). Also, it has been suggested that the metabolization of sulphur-containing amino-acids (i.e. methionine and cysteine found in animal protein) in the liver can directly cause liver injury(59). Lastly, an experimental rat study found that a high protein diet upregulated mRNA expression of genes encoding proteins involved in amino acid uptake and enhanced lipid synthesis(60). In this study, hepatic mRNA and protein levels of heat shock protein 90, a marker of liver injury, were markedly increased in these rats fed a high-protein diet. Of note, high protein intake did not result in elevated hepatic lipid concentrations in these rats.

Our study has several strengths, including a large sample size, availability of a great number of well-defined covariates, the (predefined) subgroup analyses to confirm the robustness of our findings, the evaluation of linearity and subsequent use of natural cubic splines, and the correction for multiple testing. Nonetheless, there are several considerations that need to be addressed. First, this study comprises a large predominantly elderly and Caucasian population, with a relatively alkaline diet. Despite the reassuring similar results in several subgroups of participants, caution on generalizability should be exercised. Second, due to the cross-sectional design of this study it is not possible to draw any conclusions on causality. Third, although we have corrected for a large number of different traits, we cannot exclude the possibility of residual confounding. Fourth, as with any self-administered questionnaire, the FFQ is susceptible for recall and reporter bias. Nonetheless, we have tried to limit this bias by excluding potential unreliable FFQs and by performing a sensitivity analyses with DAL-proxies corrected by the residual method to account for extraneous variation in energy intake. Fifth, one of the three included cohorts (RS III) completed the FFQ 5.5 years prior to liver imaging. We assumed that dietary data was stable over time(23), which was indeed recently shown in another paper from The Rotterdam Study(61). Nonetheless, we used study cohort as covariables in all regression models, moreover, we performed sensitivity analysis per study cohort. All the separate cohorts showed results that were comparable to the main analysis. Sixth, we used ultrasonography to assess steatosis, which has a good sensitivity to detect moderate steatosis but is poor in detecting mild steatosis and in grading steatosis. Moreover, the golden standard to assess NAFLD –and its advanced subtype non-alcoholic steatohepatitis– is a liver biopsy. However, it is unethical to perform this invasive procedure on a large scale in presumed healthy individuals. Finally, we had no dispose of acid-base biomarkers to estimate actual metabolic acidosis. However, NEAP and PRAL have been previously validated in healthy individuals and accounted for 71% of the variation in renal net acid excretion (15,25). And, although A:P has not been validated as of yet, results of A:P were very comparable to NEAP and PRAL.

In conclusion, this study shows that an acid-base (un)balanced diet is associated with NAFLD and that differences in sociodemographics, lifestyle, metabolic factors, and dietary quality did not fully explain the observed associations. Though the results should be interpreted in context of a relatively alkaline diet, our findings might explain the previously observed association of an animal protein-rich diet with NAFLD. Future research initiatives should use acid-base biomarkers, such as urinary ammonium(62), to study low-grade

metabolic acidosis more objectively. Nevertheless, dietary recommendations that are in agreement with an acid-base balanced diet, i.e. a diet rich in fruit and vegetables and poor in animal protein, are generally considered beneficial for health. Therefore, adherence to such a diet low in animal protein while awaiting the results of future studies seem justifiable.

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Ethical Approval:
All cohort participants signed a written informed consent at enrolment. The Rotterdam Study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus MC University Medical Centre Rotterdam and by the review board of The Netherlands Ministry of Health, Welfare and Sports.

Transparency declaration:
The manuscript's guarantor affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned have been explained.

Data Sharing:
Data described in the manuscript, code book, and analytic code may be made available upon request pending.

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Conflicts of Interest:
The authors declare no competing financial interests.
Authorship contributions:
LA, JKdJ & EH designed research; LA acquired participant data; JKdJ acquired nutritional data; LA, JKdJ & NE analyzed data and performed statistical analysis; LA wrote the paper; RdK provided technical support; HM & HJ obtained funding; SDM, MAI & OF supervised the study; SDM is primary responsibility for the final content. All authors read and approved the final manuscript

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Figure 1: Natural cubic splines for the association of A: PRAL, B: NEAP, and C: A:P with the predicted probability of NAFLD in (metabolic) model 3. A: Model with PRAL vs. model without PRAL (*P*=0.038). B: Model with NEAP vs. model without NEAP (*P*=0.015). C: Model with A:P vs. model without A:P (*P*=0.012). Y-axis represents predicted probability of NAFLD in (metabolic) model 3: NAFLD ~ DAL-proxy + age + gender + education level + energy intake + study cohort + smoking + units of alcohol + physical activity + HDL-cholesterol + triglycerides + metabolic syndrome + GFR + diabetes mellitus + BMI. X-axis represent values of PRAL, NEAP or A:P. The grey-colored bar represents the 95% confidence interval. The upper and lower 2.5th centile were excluded from the graphs.
Abbreviations A:P: animal protein to potassium ratio; BMI: body mass index; DAL: dietary acid load; DQ: dietary quality; GFR: glomerular filtration rate; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

Figure 2: Associations of DAL-proxies with NAFLD stratified by various covariates in the metabolic model. Values are odds ratios of the fourth quartile with 95% confidence intervals taking the first quartile as reference. All strata are analysed within modified (metabolic) model 3: NAFLD ~ DAL-proxy + (age) + (gender) + education level + energy intake + (study cohort) + smoking + units of alcohol + physical activity + HDL-cholesterol + triglycerides + metabolic syndrome + (GFR) + diabetes mellitus + (BMI). This figure represents data that can also be found in more detail in *Supplementary Table 4 – 9.*
Abbreviations A:P: animal protein to potassium ratio; BMI: body mass index; DAL: dietary acid load; GFR: glomerular filtration rate; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

Table 1: Characteristics per quartile PRAL

	PRAL				
	Quartile 1	**Quartile 2**	**Quartile 3**	**Quartile 4**	***P*-value**
DAL					
PRAL	-24.4 (-33.7 ; -19.1)	-9.7 (-12.5 ; -7.2)	-0.33 (-2.3 ; 1.9)	11.1 (7.4 ; 17.6)	n/a
NEAP	25.6 (21.9 – 28.4)	32.7 (30.9 – 34.3)	38.7 (37.0 – 40.7)	47.4 (44.1 – 52.9)	<0.001
A:P	9.2 (7.6 – 10.5)	11.7 (10.4 – 13.2)	13.9 (12.4 – 15.4)	17.2 (15.0 – 19.9)	<0.001
Demographics					
Age	70.2 (8.8)	69.8 (8.6)	69.8 (8.8)	69.1 (9.2)	0.057
Female (%)	67.6	62.5	54.9	48.2	<0.001
Caucasian (%)	97.6	97.9	97.8	97.2	0.804
Education Level (%)					
Low	48.4	49.7	47.9	47.7	0.167
Intermediate	27.7	29.4	31.6	32.5	
High	23.9	20.9	20.5	19.8	
Smoking status (%)					
Never	39.5	37.7	34.5	32.6	0.009
Current / Former	60.5	62.3	65.5	67.4	
Alcohol (units/d)	0.45 (0.04 – 1.16)	0.49 (0.08 – 1.22)	0.45 (0.07 – 1.22)	0.43 (0.03 – 1.17)	0.203

Physical Activity (METh/wk)	46.4 (18.5 – 84.5)	43.0 (17.0 – 78.8)	37.0 (14.3 – 74.5)	34.6 (13.5 – 73.2)	<0.001
Energy intake (kcal/d)	2175 (1823 – 2667)	1929 (1557 – 2363)	1891 (1488 – 2360)	2105 (1700 – 2668)	<0.001
Physical examination					
BMI (kg/m^2)	26.5 (24.3 – 29.4)	26.8 (24.5 – 29.4)	27.0 (24.6 – 29.9)	27.2 (24.7 – 30.0)	0.010
Biochemistry					
AST (U/L)	24 (21 – 28)	24 (21 – 28)	24 (21 – 28)	25 (21 – 29)	0.125
ALT (U/L)	18 (14 – 24)	18 (14 – 23)	18 (14 – 24)	20 (15 – 25)	<0.001
GGT (U/L)	21 (16 – 30)	22 (16 – 32)	23 (17 – 34)	25 (18 – 37)	<0.001
Platelets (*10^9/L)	269 (232 – 309)	263 (224 – 305)	259 (220 – 303)	257 (218 – 303)	0.002
HOMA-IR	2.4 (1.7 – 3.7)	2.5 (1.7 – 3.9)	2.6 (1.7 – 4.2)	2.9 (1.8 – 4.6)	<0.001
Total Cholesterol (mmol/L)	5.6 (1.1)	5.5 (1.1)	5.4 (1.1)	5.3 (1.1)	<0.001
HDL-C (mmol/L)	1.5 (0.4)	1.5 (0.4)	1.5 (0.4)	1.4 (0.4)	<0.001
Triglycerides (mmol/L)	1.3 (1.0 – 1.7)	1.3 (1.0 – 1.7)	1.3 (1.0 – 1.7)	1.3 (1.0 – 1.8)	0.482
GFR	76 (66 – 86)	76 (66 – 86)	75 (66 – 85)	76 (65 – 86)	0.796
Comorbidities					
Metabolic Syndrome	48.4	49.7	54.2	57.1	0.003
- Waist circumference	40.2	43.2	43.8	45.6	0.112
- Triglycerides	41.6	45.4	48.4	48.5	0.007
- HDL-Cholesterol	40.1	44.7	46.5	47.2	0.007
- Blood pressure	84.6	85.0	83.7	83.7	0.794
- Fasting Glucose	41.8	45.1	46.6	53.5	<0.001
Diabetes Mellitus (%)	11.1	11.6	12.5	17.3	<0.001
Hypertension (%)	73.8	75.7	72.1	74.3	0.363
NAFLD	30.9	31.8	35.2	39.9	<0.001

Data is expressed as mean (SD), median (P25-P75) or percentage. **P*-value is based on ANOVA, Kruskall-Wallis test or Chi-square test.

Abbreviations ALT: alanine aminotransferase; A:P: animal protein to potassium ratio; AST: aspartate aminotransferase; BMI: body mass index; DAL: dietary acid load; GFR: glomerular filtration rate; GGT: gamma-glutamyltransferase; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

Table 2: Correlations between DAL-proxies and diet

	PRAL	**NEAP**	**A:P**
	r_s	r_s	r_s
Total protein (g)	0.17	0.23	0.21
Animal protein	0.26	0.32	0.51
Vegetable protein	- 0.08	- 0.03	- 0.31
Total carbohydrates (g)	- 0.27	- 0.23	- 0.32
Mono-and disaccharides	- 0.45	- 0.42	- 0.35
Polysaccharides	0.02	0.06	- 0.20
Fiber	- 0.40	- 0.36	- 0.48
Total fat (g)	0.20	0.25	0.12
Saturated fat	0.24	0.27	0.21
Mono-unsaturated fatty acids	0.20	0.25	0.12
Poly-unsaturated fatty acids	0.11	0.16	- 0.04
Trans fatty acids	0.23	0.24	0.18
Minerals (mg)			
Vitamin E	- 0.03	0.03	- 0.09
Magnesium	- 0.23	- 0.18	- 0.26
Potassium	- 0.44	- 0.39	- 0.30
Phosphorus	0.05	0.08	0.08
Calcium	- 0.05	- 0.03	0.06
DQ score (points)	- 0.29	- 0.29	- 0.32
↑Vegetables	- 0.27	- 0.23	- 0.24
↑Fruit	- 0.51	- 0.50	- 0.37
↑Whole grain products	0.09	0.10	- 0.12
↑Legumes	- 0.03	- 0.01	- 0.08
↑Nuts	0.03	0.04	- 0.09
↑Dairy	- 0.02	- 0.04	0.09
↑Fish	0.04	0.08	0.14
↑Tea	- 0.05	- 0.05	- 0.05
↑Whole/Refined grains	- 0.04	- 0.06	- 0.13
↑Unsaturated fats/oils	- 0.06	- 0.05	- 0.06

↓Red and processed meat	- 0.17	- 0.22	- 0.32
↓Sugar containing drinks	0.07	0.06	0.06
↓Alcohol	- 0.02	- 0.01	- 0.02
↓Salt	- 0.22	- 0.25	- 0.13

By Spearman's rank correlation. DQ score can theoretically vary from 0-14 points. DQ subtypes are dichotomous.

Legend Spearman correlation:

negative			positive		
moderate	**weak**	**very weak**	**very weak**	**weak**	**moderate**
-0.59 to -0.40	-0.39 to -0.20	-0.19 to -0	0 to 0.19	0.20 to 0.39	0.40 to 0.59

Abbreviations A:P: animal protein to potassium ratio; DAL: dietary acid load; NEAP: net endogenous acid production; PRAL; potential renal acid load.

Table 3: Logistic regression analyses of DAL-proxies with NAFLD as dependent variable

Total population (n=3882)			
	Q2	**Q3**	**Q4**
PRAL n per quartile	**(n=970)**	**(n=971)**	**(n=970)**
NEAP n per quartile	**(n=970)**	**(n=971)**	**(n=970)**
A:P n per quartile	**(n=970)**	**(n=971)**	**(n=970)**
Model 1 (sociodemographic)			
PRAL	0.99 (0.81 – 1.20)	1.14 (0.94 – 1.38)	**1.42 (1.17 – 1.72)†**
NEAP	1.02 (0.84 – 1.24)	**1.24 (1.02 – 1.50)**	**1.40 (1.15 – 1.69)†**
A:P	1.14 (0.94 – 1.39)	**1.36 (1.12 – 1.65)†**	**1.63 (1.35 – 1.97)†**
Model 2 (lifestyle)			
PRAL	0.98 (0.81 – 1.19)	1.12 (0.92 – 1.36)	**1.38 (1.14 – 1.67)†**
NEAP	1.03 (0.85 – 1.25)	**1.22 (1.01 – 1.48)**	**1.36 (1.12 – 1.65)†**
A:P	1.14 (0.94 – 1.39)	**1.33 (1.09 – 1.61)†**	**1.58 (1.31 – 1.92)†**
Model 3 (metabolic)			
PRAL	0.97 (0.77 – 1.21)	1.09 (0.87 – 1.36)	**1.26 (1.01 – 1.58)**
NEAP	0.97 (0.78 – 1.22)	1.18 (0.94 – 1.47)	1.24 (0.99 – 1.56)
A:P	0.96 (0.77 – 1.21)	1.09 (0.87 – 1.36)	1.22 (0.97 – 1.52)
Model 4 (metabolic + DQ)			
PRAL	0.97 (0.77 – 1.21)	1.09 (0.87 – 1.37)	**1.27 (1.01 – 1.60)**
NEAP	0.98 (0.78 – 1.22)	1.18 (0.94 – 1.47)	1.25 (0.99 – 1.57)
A:P	0.96 (0.77 – 1.21)	1.09 (0.87 – 1.37)	1.22 (0.97 – 1.54)

Values are odds ratios with 95% confidence intervals taking quartile 1 as reference. Bold values indicate P<0.05. † Indicates significant values using P < 0.028 as determined by Sidák.
Model 1 (socio-demographic) is adjusted for age, gender, education level, energy intake and study cohort.
Model 2 (lifestyle) is in addition previous model adjusted for past or current smoking, units of alcohol, and physical activity .
Model 3 (metabolic) is in addition to the previous model adjusted for HDL-cholesterol, triglycerides, metabolic syndrome, GFR, diabetes mellitus and BMI.
Model 4 (metabolic + DQ) is in addition to the previous model adjusted for DQ.
Abbreviations A:P: animal protein-potassium ratio; DQ: Dietary Quality; NEAP: net endogenous acid production; PRAL: potential renal acid load; Q: Quartile.
Abbreviations A:P: animal protein to potassium ratio; BMI: body mass index; DAL: dietary acid load; DQ: dietary quality; GFR: glomerular filtration rate; HDL: high density lipoprotein; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.